# Mars Sample Return Planning Sterilization Working Group 2nd Meeting

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# Proteinaceous Particles Tests

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# Background



## Rationale for Tests

- Draft Protocol from NASA-led workshop "Detection of Possible Biohazards in Martian Samples Returned to Earth" (October 2002)
  - "An effort should be made to characterize species from environments as similar as possible to those on Mars, and these species will be used to validate a sterilization process"
- Space Studies Board of the National Research Council (1997)
  - Asked by NASA to address the following concerns: "The potential for a living entity to be included in a sample to be returned from another solar system body, in particular Mars; Scientific investigations should be conducted to reduce uncertainty in this assessment"
- Mars Sample Return Sterilization Workshop, (Jan. 30-31, 2019)
  - NASA Planetary Protection Officer and Committee has recommended "proteins/prion surrogates be included in that mixed community of BI's"

- Address backward contamination
  - Necessity for technology development
  - Increased knowledge of life as we know it and will need to develop a biological indicator to cover the spectrum of potential contaminants
- JPL Biotechnology and Planetary Protection Group have used industry based lethality testing with spore biological indicators
  - Past mission requirements focused on hardy spore formers
- We are presented with a unique environment in which to perform sterilization

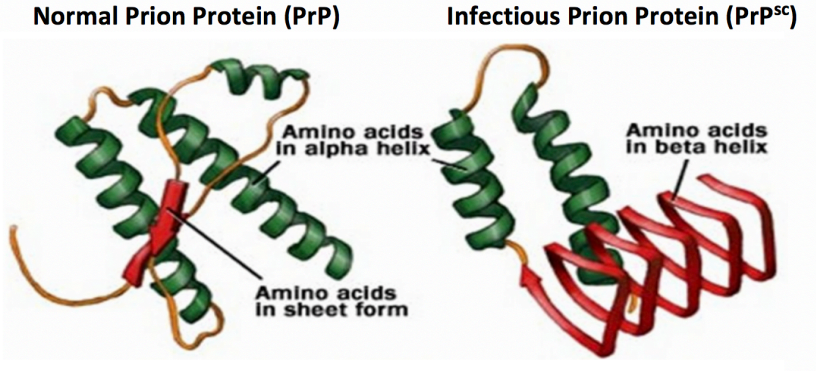
# Introduction: What are Proteinaceous Particles?

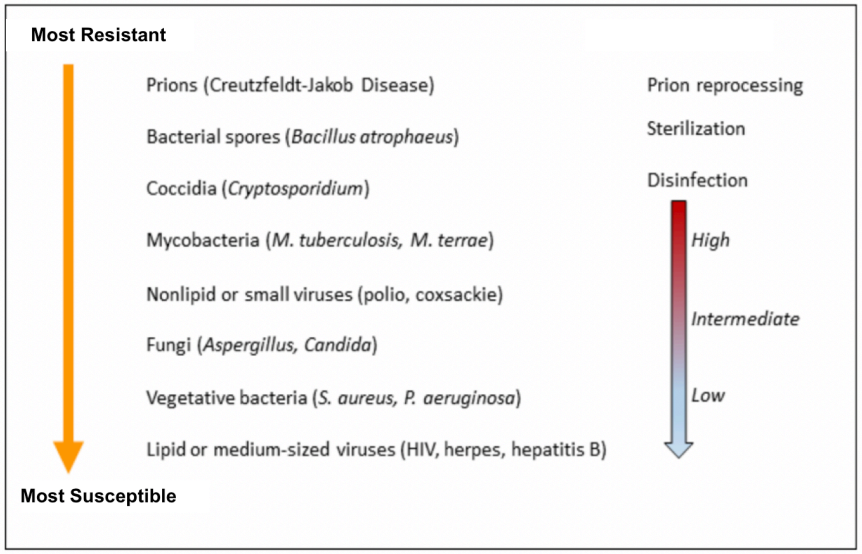


- Proteinaceous particles (prions) are self-propagating misfolded proteins that are capable of accumulating into abnormal filaments composed of beta-sheets called amyloids
- Source of environmental contamination capable of transfer to mammals as an infectious agent
- No genetic material such as DNA and RNA
- Prion inactivation requires denaturation of the protein into a non-functional form

**Why is prion inactivation a challenge?**

- Lack of research knowledge
- Risk as a potential health hazard
- Traditional decontamination methods target RNA and DNA therefore effectiveness on prions has not been the primary focus




Modified from Russell and Favero.[13, 344]

1. Liebman SW, Chernoff YO. Prions in yeast. Genetics. (2012);191(4):1041–1072. doi:10.1534/genetics.111.137760.
2. Chernoff, Y.O., Galkin, A.P., Lewitin, E., Chernova, T.A., Newnam, G.P. & Belenkiy, S.M. (2000) Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein. Mol. Microbiol. 35, 865–876.

# Proposition



1. This work will drive the development of proteinaceous particles as a biological indicator

2. The biological indicators would be used to develop, test and validate the MSR sterilization system

# Objectives



1. Identify surrogate prion BI's that are stable

2. Evaluate efficacy of sterilization modalities against selected prion biological indicator

3. Establishment of parameters using tests that define yeast prion sterilization (conformational change and inactivation)

# Rationale for Candidate Selection



- From the previous workshop, Gary Ruvkun had suggested using a surrogate prion
- A non-pathogenic yeast strain, *Saccharomyces cerevisiae*, is capable of producing prion proteins and sheets of aggregated protein
  - There are a handful of yeast-prions that have been discovered however, Sup35 has been characterized most extensively
- **Advantages for yeast prions models**:
  - Cost effective
  - Less invasive method that will replace animal injections
  - Safer alternative to human prions

- Yeast prion, **Sup35p** has been used to understand protein aggregation and evolution of yeast characteristics

| | Yeast Prion ($Sup35p^{PSI+}$) | BSE Prion ($PrP^{Sc}$) |
|---|---|---|
| Solubility | Insoluble (aggregate) | Insoluble (aggregate) |
| Protease (common conditions) | Resistant | Resistant core |
| Structure | Rich in β-sheet | Rich in β-sheet |
| Polymerization | Yes | Yes |
| Isoform (normal cellular form) | Monomer, Sup35p | Monomer, $PrP^{C}$ |
| Characteristic Sequence | 5 imperfect repeats (PQGGYQQYN) | 5 copies (PHGGGWGQ) |
| Infectivity | Infectious, non-pathogenic | Infectious, pathogenic |

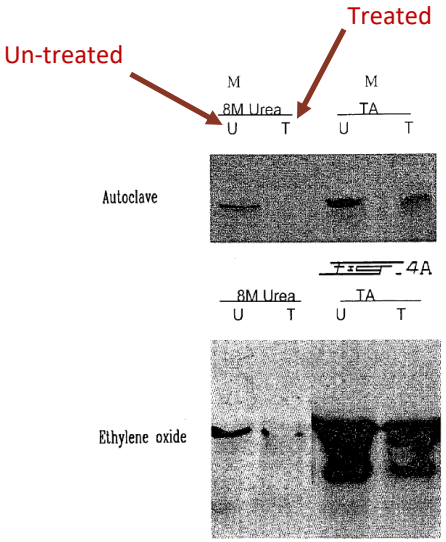
**Table 1.** Comparison of the characteristics of Yeast prion ($Sup35p^{PSI+}$) and BSE Prion or known as the mammalian prion ($PrP^{sc}$) (Rojanatavorn, 2004)

3. Rojanatavorn, K. (2004) Production and Enzymatic Degradation of Sup35NM, a Prion-Like Protein from Yeast . North Carolina State University, Raleigh, North Carolina.

# Current state-of-the-art Sterilization Systems for Yeast Prions



- Validated technologies for sterilization and inactivation of yeast prions includes:
  - Pre-heating at 80ºC to 115ºC with protease
  - Autoclaving (121ºC) with Urea
  - Ethylene oxide treatments with Urea
- Efficacy of sterilization and inactivation of prion surrogate proteins remains a knowledge gap
- There are a number of sterilization modalities which have been identified as potential candidates for sterilization of Mars material, however for preliminary testing, dry heat will be considered for testing with surrogate prions




Sterilization cycles used for the evaluation of the degradation of Sup35p

| Sterilization process | Sterilizing agent | Cycle | Time required for complete cycle |
|---|---|---|---|
| Autoclave | Heat | Temperature: 121° C.<br>Pressure: 1 atm | 1 hour |
| Sterivac ®, 3M | Ethylene Oxide (EO) | (a) Temperature: 134° F.<br>(b) Preheating time: 30 min.<br>(c) Sterilization time: 2 h10<br>(d) Ventilation time: 12 h | Approx. 16 hours |

Table 2 provides the parameters for the patent tests. The top figure demonstrates that the samples Sup35p in TA were analyzed with Western blot samples were resistant to both autoclave and ethylene oxide treatments (Belhumeur et al.,2000).

4. Belhumeur et al. (2000) Patent: World Organization Patent No. WO 0065344A3 Al. Retrieved from https://patents.google.com/patent/WO2000065344A3.

# Preparation and Initial Tests



**Obtain and Confirm Aggregated prion proteins with Thioflavin T**

Confirm Protein Aggregation:

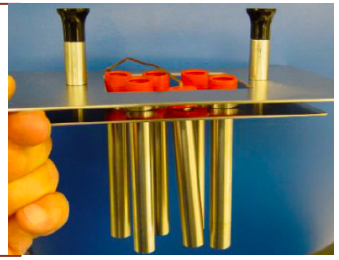
- The amyloid fibrils can be readily detected using **Thioflavin T (ThT)**, a small molecule that gives strong fluorescence upon binding to amyloids.

**Prepare proteins on metallic coupons**

- Protein preparations will be spotted onto metallic coupons (0.1 ug to 0.5 ug)
- Proteins will be desiccated

**Heat Exposure and Recovery**

- Prepared individual coupons will be placed into a Thermal Spore Exposure Vessel (TSEV) and heated in a constant high temperature silicone oil bath.
- The samples will be exposed to (121-145ºC). Heat exposures will be determined based on the central value for the range of heat inactivation and will be adjusted based on existing literature and past work on prions. Coupons are placed in non-denaturing buffer to recover proteins

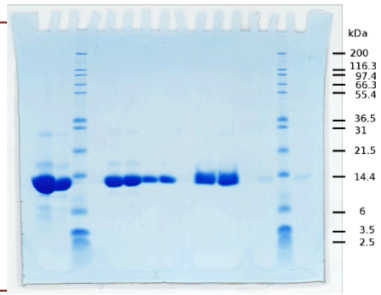


**SDS-PAGE**

**S**odium **D**odecyl **S**ulphate-**P**olyacrylamide **G**el **E**lectrophoresis

**Compare results to the control band**

- Change in size (multi-bands)
- Change in shape (change in band location)
- Change in charge (change in band location)
- No change

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6. Edgeworth, J. A., Sicilia, A., Linehan, J., Brandner, S., Jackson, G. S., & Collinge, J. (2011). A standardized comparison of commercially available prion decontamination reagents using the Standard Steel-Binding Assay. *The Journal of general virology*, *92*(Pt 3), 718–726. doi:10.1099/vir.0.027201-0

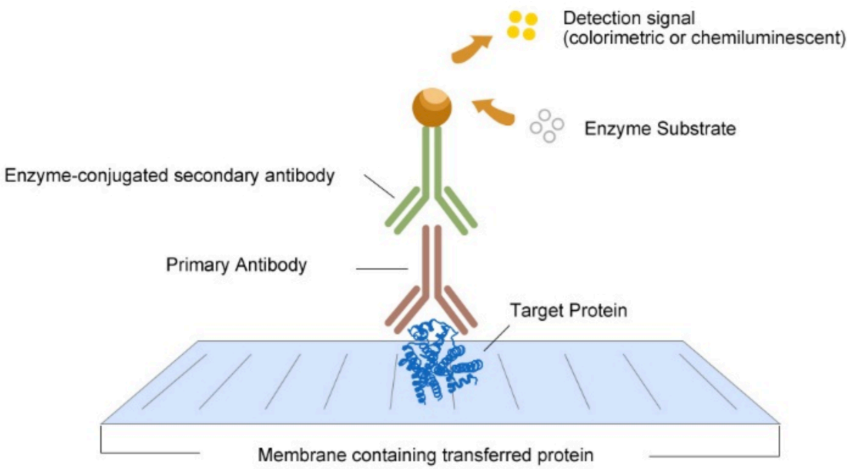
# Conformational Change Tests



Western Blot → Change in conformation (no bands)

Western Blot → No conformational change (same band position)

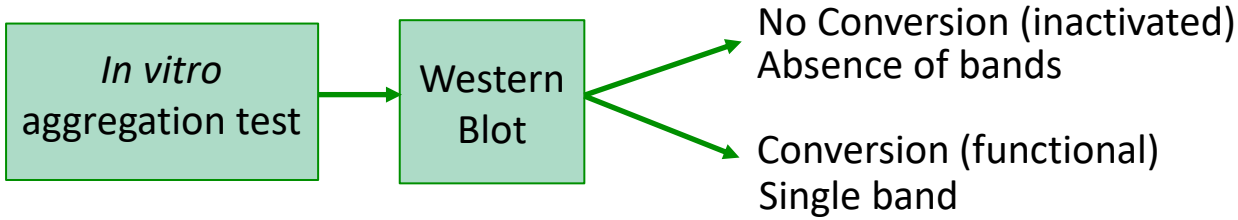
- Western Blot is a test with high specificity to visualize the effects for sterilization of prion surrogate samples.
- Western Blot is currently the **gold standard** for protein testing and has higher specificity when compared to other immunoassay methods such as ELISA.
- The protein is separated based on molecular weight and or charge by electrophoresis in a gel matrix, and transferred to a membrane and probed with **antibody specific to the protein** of interest.
- If the conformation of the protein has changed, the antibody will not bind and the protein band will not be detected on the western blot.




7. Mahmood T, Yang PC. (2012) Western blot: technique, theory, and trouble shooting. N Am J Med Sci. 2012;4(9):429–434. doi:10.4103/1947-2714.100998.

# Inactivation Tests






- The modality exposed Sup35$^{psi+}$ (prions) will be mixed with the lysates of Sup35$^{psi-}$ in sucrose to **observe for functionality**
- As controls, lysates of Sup35$^{psi-}$ will be incubated for two hours without addition of Sup35$^{psi+}$
- Western Blots will determine if modality exposed Sup35$^{psi+}$ has converted Sup35$^{psi-}$ to a prion-like state.

**Absence of bands** indicates that the modality exposed Sup35psi+ **does not have the ability to self-propagate** and will not convert Sup35psi-.

**Presence of bands** indicates that the modality exposed Sup35psi+ has the **ability to self-propagate** and convert Sup35psi-.

8. Paushkin, S.V., Kushnirov, V.V., Smirnov, V.N., & Ter-Avanesyan, M.D. (1997). In vitro propagation of the prion-like state of yeast Sup35 protein. *Science, 277 5324*, 381-3.

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# Summary of Proposed Tests



## 1. Preparation and Initial Tests

Obtain and Confirm Aggregated prion proteins with Thioflavin T → Prepare on metallic coupons → Heat Exposure and Recovery → SDS-PAGE →

- Change in size (multi-bands)
- Change in shape (change in band location)
- Change in charge (change in band location)
- No change

## 2. Conformational Change Tests

Western Blot →
- Change in conformation (absence of bands)
- No conformational change (same band position)

## 3. Inactivation Tests

*In vitro* aggregation test → Western Blot →
- No Conversion (inactivated) Absence of bands
- Conversion (functional) Single band

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# Summary



- This particular area of focus is one which has been identified as a necessary component in the development of sterilization strategies associated with a potential Mars Sample Return effort

- This will be the first known study to define parameters for sterilization testing against prion surrogate proteins

- Although there are a number of sterilization modalities which have been identified as potential candidates for the sterilization of Mars material (UV, chemical, heat, VHP, plasma, and gamma radiation), their efficacy in the sterilization/inactivation of prion surrogate proteins is unknown and to our knowledge remains untested.

- **Therefore this method would need to be developed as part of the sterilization plan**

# References




1. Liebman SW, Chernoff YO. Prions in yeast. Genetics. (2012);191(4):1041–1072. doi:10.1534/genetics.111.137760.
2. Chernoff, Y.O., Galkin, A.P., Lewitin, E., Chernova, T.A., Newnam, G.P. & Belenkiy, S.M. (2000) Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein. Mol. Microbiol. 35, 865–876.
3. Rutala WA, Weber DJ. (2001) Creutzfeldt-Jakob Disease: Recommendations for Disinfection and Sterilization. Clin Infect Dis. 32:1348–1356. doi: 10.1086/319997.
4. WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies: Report of a WHO Consultation, Geneva, Switzerland, 23-26 March 1999.
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# Back-Up Slides

# Renaturation



- What is renaturation?
  - After prion inactivation, the denatured prions would have the ability to renature or restore infectivity by refolding.

- Prion protein renaturation has not been shown to occur once denaturation has been achieved
- Study performed by Prusiner, attempted to restore infectivity of mammalian prions after exposure to denaturants such as chaotropic salts and as a reverse treatment, subjected the samples to dialysis to remove the salts while incubating the samples.
  - **Renaturing conditions will require complex laboratory manipulations** and will not be performed during acquisition of the prion samples

11. Pruisiner, S et al. (1993) Attempts to restore scrapie prion infectivity after exposure to protein denaturants. Proceedings of the National Academy of Sciences. 90 (7) 2793-2797; DOI:10.1073/pnas.90.7.2793.

# Collaborators



- Experts in Prion Proteins
  - Instrumental in the development of assay protocols and associated tests to confirm conformational change and inactivation
  - Personnel available and will support questions for processing prion proteins

- Collaborators:
  - Georgia Institute of Technology:
    - **Dr. Yury O. Chernoff** is a professor and the Director for the Center for Nanobiology of Macromolecular Assembly Disorders (NanoMAD). His research focuses on the development of yeast models to study prions and amyloids. Dr. Chernoff has over 30 years of experience and established evolutionary conservation of prion-forming properties, developed yeast models for prion species barrier and for aggregation/toxicity of mammalian proteins, and uncovered the role of cytoskeletal networks and proteolytic pathways in prion and amyloid phenomena.

  - Colorado State University
    - **Dr. Eric Ross** is a professor for the Department of Biochemistry and Molecular Biology. He has Over 15 years of experience studying yeast prions. He has worked extensively with the prion proteins Sup35 and Ure2, both in yeast and in-vitro. He has done pioneering research defining how the amino acid sequence of prion proteins contributes to the formation and stability of prion aggregates, and contributed to the development of computational models to examine the kinetics of prion aggregation.

# Risk factors for Proteinaceous Particles



- Associated with higher life or eukaryotic life
  - Therefore probability is low that they would be encountered on Mars
- Low amount of organic material in Mars regolith (estimate value?)
- Not abundant

# Alternative Surrogates



Alternatives surrogate proteins used in parallel with yeast prions include

- RNase A
- Urease
- Malic dehydrogenase
- myoglobin
- Bovine Serum Albumin

These proteins are known to fold and form aggregates

TABLE 3. Inactivation of viruses, viroids, prions, and enzymes by irradiation at 254 nm

| Particle | $D_{37}$ (J/m$^2$) | Reference |
|---|---|---|
| Bacteriophages | | |
| T2 | 4 | 18 |
| M13 | 6.5 | This paper |
| M13 | 9.9 | 30 |
| S13 | 20 | 15 |
| $\phi\chi$174 | 20 | 15 |
| Rous sarcoma virus | 150 | 12 |
| Polyomavirus | 240 | 17 |
| Friend leukemia virus | 500 | 16 |
| Murine leukemia virus | 1,400 | 19 |
| *Eco*RI site | 2,750 | This paper |
| PSTV | 5,000 | 9 |
| PSTV | 4,800 | This paper |
| Fumarase | 3,100 | 13 |
| Glutamic dehydrogenase | 4,700 | 13 |
| RNase A | 11,800 | This paper |
| Scrapie prions (purified) | 17,000–22,000 | This paper |
| Scrapie prions (in 10% brain homogenate) | 24,000 | 19 |
| Urease | 30,000 | 13 |
| Malic dehydrogenase | 41,000 | 13 |

# Rationale for Tests



1. Protein replication differs from cellular organism replication, making quantification difficult and therefore will require molecular assays for tests.
2. According to the World Health Organization:

   "Aggressive sterilization strategies for mammalian prions such as chemical and physical methods have proven insufficient for inactivation."

3. CDC and WHO guidelines recommended heat exposure at 135°C with sodium hydroxide. The focus has since shifted from prion sterilization to these recommended methods.
4. We are presented with a unique environment in which to perform sterilization.

**Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008**

3. Rutala, William A. et al. (2008) CDC Guideline for Disinfection and Sterilization in Healthcare Facilities. Retrieved from https://www.cdc.gov/infectioncontrol/pdf/guidelines/disinfection-guidelines.pdf.
4. WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies: Report of a WHO Consultation, Geneva, Switzerland, 23-26 March 1999.

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